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Environmental effects on zein chromatograms of maize inbred lines revealed by reversed-phase high-performance liquid chromatography

J. S. C. Smith and O. S. Smith

Departments of Research Specialists and Data Management, respectively, Pioneer Hi-Bred International, Inc., 7301 NW 62nd Avenue, Johnston, IA 50131, USA

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Summary. Alcohol soluble seed storage proteins (zeins and alcohol soluble glutelins) of maize (*Zea mays* L.) were separated by reversed-phase high-performance liquid chromatography (RP-HPLC). The objectives were to assess the reproducibility of chromatographic profiles using seed of inbred lines that had been produced in different locations and years. Reproducible differences between sources were seen but these were restricted to proteins that contributed 2% or less to an inbred profile. The majority of variation (93% for peak percent area; 99.8% for elution time) was between inbreds. RP-HPLC can therefore provide distinctive phenotypic profiles that are largely characteristic of genotype. Such qualitative and quantitative data will be valuable for studies of taxonomy, evolution, genetics, and germplasm identification.

Key words: Taxonomy – Germplasm identification – Varietal identity – Environmental interaction – Genetics

Introduction

Characterization of germplasm is a prerequisite for the establishment of line or cultivar purity, the investigation of relationships among lines, and for the identification of genetic markers that could allow more efficient gene transfer and selection.

Reversed-phase high-performance liquid chromatography (RP-HPLC), a procedure that separates proteins on the basis of hydrophobicity (Bietz 1983), has allowed cultivar characterization of wheat (*Triticum aestivum* L., *T. durum* Desf.), (Burnouf et al. 1983; Bietz et al. 1984a, b; Burnouf and Bietz 1984), barley (*Hordeum vulgare* L.) (Marchylo and Kruger 1984), and maize (*Zea mays* L.) (Bietz 1983). RP-HPLC has revealed protein components that are associated with grain quality in wheat (Burnouf and Bietz 1984) and in maize (Paulis JW, personal communication). In respect of cultivar identification, the majority of RP-HPLC studies have utilized alcohol soluble proteins (prolamins and glutelins).

A critical factor that determines the effectiveness of any technique to provide cultivar identification is the extent to which the expression of the identifying characters is affected by the environment. If significant environmental effects are present, the phenotype, in the case of RP-HPLC the chromatogram, will not characterize a genotype in a consistent or readily interpretable manner.

There are several factors pertaining to cereal proteins and their analysis by RP-HPLC that mandate an examination of possible environmental effects. These are 1) the heterogenic nature of cereal proteins (Bietz 1983; Landry et al. 1983; Wall et al. 1984; Wilson 1984); 2) the exceptionally high resolving power of RP-HPLC (Bietz 1983); 3) the qualitative and quantitative nature of data obtained from RP-HPLC analysis (Marchylo and Kruger 1984); 4) the qualitative and quantitative effects of environment on seed protein content (Higgins 1984); and 5) possible unpredictable interactions mediated through the complex genetic organization of zein genes and regulatory genes (Soave and Salamini 1983). This study, therefore, reports upon the stability of RP-HPLC chromatographic profiles of prolamin (zein) and glutelin for inbred lines of maize grown at diverse locations and across different seasons.

Materials and methods

Seed that was planted to produce the sources analyzed in this study could be traced back within, at most, three generations to foundation stocks that previously had been self-pollinated by hand for at least six generations and which had been shown to be morphologically uniform in field growout trials. Inbred

lines, proprietary material of Pioneer Hi-Bred International, Inc., and locations of seed increase in isolated production plots, with a minimum separation of 200 m from adjacent maize fields, are listed in Table 1. Seed was stored at 6 °C following harvest. These inbred lines represent a diverse array of elite U.S. germplasm encompassing "Iowa Stiff Stalk Synthetic", "Lancaster Sure Crop", and unrelated pedigree source materials.

For an initial experiment, two extracts were prepared from each of two sources for inbreds O51 and G39. Subsequently, one extract of each inbred was prepared for analysis. Twenty to 25 kernels of each sample were ground to flour in an Udy cyclone hammermill and nondefatted flour was extracted by vigorous agitation in a 1:5 (w/v) ratio of 2-propanol (55%), water (44%), and 2-mercaptoethanol (1%) (Wilson 1984) for 15 h at 25 °C. The extract, therefore, contained reduced zein and alcohol soluble glutelins (Landry et al. 1983). Extracts were centrifuged at 12,000 g for 20 min. The supernatant was further centrifuged at 13,000 g for 2 min, and either analyzed directly or stored at −70 °C. Control samples containing approximately 10 and 100 fold the amount of captan and treatment dyes that are present in commercial seed stocks were prepared in an identical fashion.

Twenty µl of extract were analyzed using a Brownlee reversed-phase RP 300 C_8 chromatography column at 70 °C. Solvent pumps, system controller, and spectrophotometer were supplied by Waters Chromatography, Div. Millipore. Chromatographic running conditions were from 49% (v/v) acetonitrile (ACN), 51% (v/v) water, plus 0.1% (v/v) trifluoroacetic acid (TFA) at extract injection time, to 58% (v/v) ACN, 42% (v/v) water, plus 0.1% TFA with a linear gradient lasting 40 min. Protein was detected spectrophotometrically at 210 nm. Peak areas were calculated using a chromatography program supplied by Nelson Analytical, Inc., and all data were stored directly on disc. Peak areas were normalized by disregarding all peaks eluting prior to 5 min (solvent peaks) and then expressing peak areas as a percentage of the remaining chromatogram. Separations were terminated at 30 min after sample injection.

Peak numbers were assigned to the peaks for each sample such that the same peaks from different samples of a particular genotype had the same number. Peaks from two samples were considered to be the same peak if they eluted within 10 s of each other. An analysis of variance was performed on the traits; 1) time (of peak appearance) and 2) area using a completely nested model. Sources of variation were computed for differences among peaks (peak); inbreds within peak (inbred); seed source within each inbred pooled over peaks (sources); and differences among duplicate extracts (when available) within each source pooled over inbreds and peaks (extract). The amount of variation due to inbreds within peaks, seed sources, and duplicate extracts was expressed as the percentage of the sum of these three terms. The variation among peaks is a function of the proteins and is not pertinent to the objectives of this study.

Table 1. Provenance data for the samples of inbred lines analyzed by HPLC. All samples, except G39-5 and G50-4 were treated with captan and dye

Inbred	Sample no.	Production location	Year
051	051-1	Pelee Island, Ontario	1979
	051-2	Hawaii	1979
	051-3	Pelee Island, Ontario	1981
	051-4	Pelee Island, Ontario	1980
207	207-1	Grand Junction, CO	1979
	207-2	Pelee Island, Ontario	1979
	207-3	Weslaco, TX	1980
	207-4	Pelee Island, Ontario	1981
G35	G35-1	Hawaii	1981
	G35-2	Johnston, IA	1981
	G35-3	Pelee Island, Ontario	1982
G39	G39-1	Johnston, IA	1982
	G39-2	Pelee Island, Ontario	1983
	G39-3	Pelee Island, Ontario	1982
	G39-4	Weslaco, TX	1982
	G39-5	Homestead, FL	1983
G50	G50-1	Johnston, IA	1983
	G50-2	Pelee Island, Ontario	1983
	G50-3	Hawaii	1983
	G50-4	Homestead, FL	1982

Results

All peaks from control samples, which contained extraction solvent, captan, and dyes, eluted together with the solvent front, prior to 5 min following sample injection. Analysis of variance data from the initial experiment in which replicate extracts were analyzed are given in Table 2. The level of variation attributable to the pooled variation among extracts (extract) was low for both area percentage and elution time. Pooled variation among sources of a given inbred was higher for both percent area and elution time, but the majority of variation in both percent area and elution time was among inbreds. Figures 1 and 2 present chromatograms

Table 2. Analysis of variance and variance components combined over inbreds 051 and G39 for standarized areas and elution times

Percent area

Source of variation	df	Mean square	Variance component	% Total
Peak	29	28.02**	6.21	
Inbred	30	170.26**	19.61	88.1
Source	60	99.84**	2.17	9.7
Extract	180	5.92	0.49	2.2
Total	299	28.02		

Elution time

Source of variation	df	Mean square	Variance component	% Total
Peak	29	1,717,507.70**	171,621.8	
Inbred	30	1,191.98**	225.2	80.4
Source	60	114.99**	40.1	14.3
Extract	180	14.79	14.8	5.3
Total	299			

** F value significant at 1% level

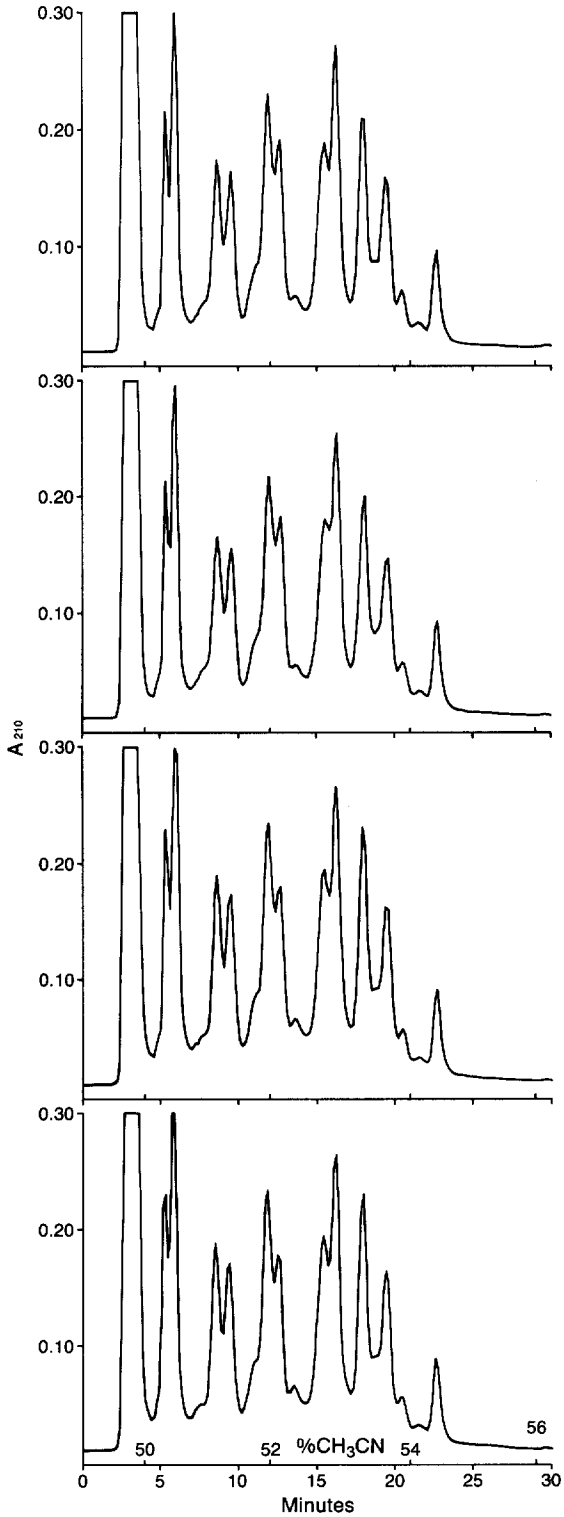

Fig. 1. Chromatograms of 2 separate extracts of 2 different sources of inbred O51; source O51-2 the two lower and source O51-3 the two upper chromatograms, respectively

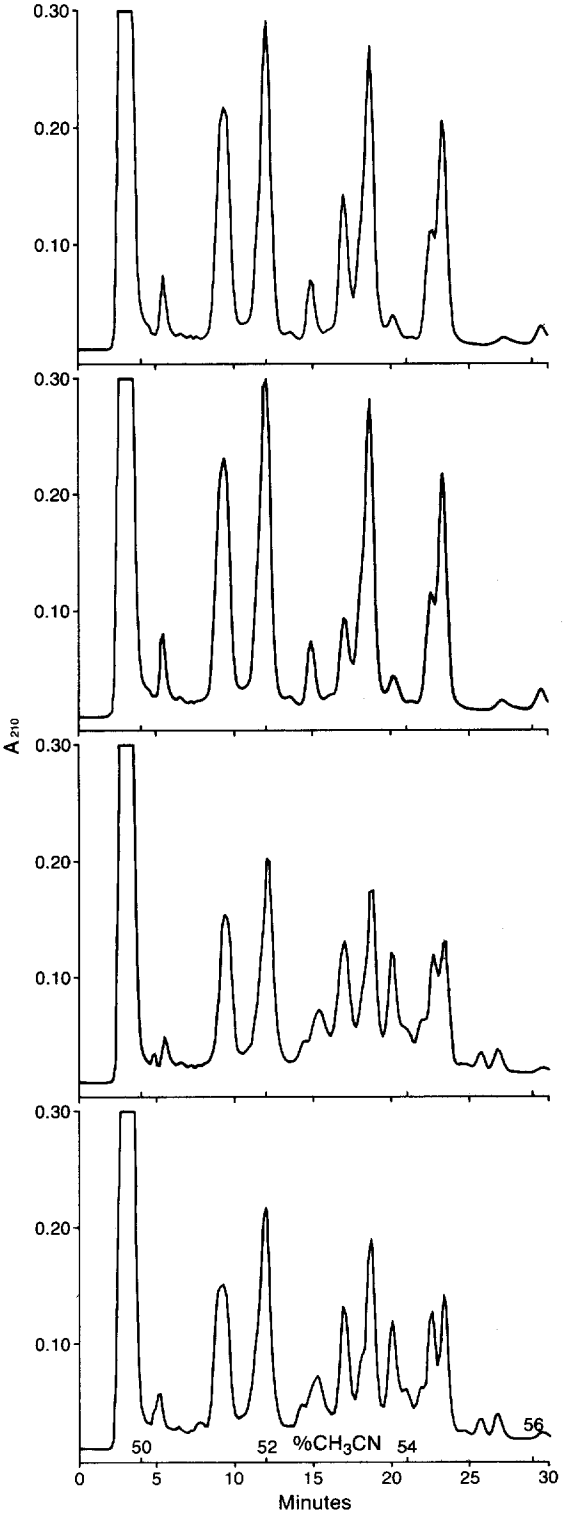

Fig. 2. Chromatograms of 2 different extracts of 2 sources of inbred G39; source G39-3 the two lower and source G39-5 the two upper chromatograms, respectively

Table 3. Analysis of variance components form chromatograms of 5 inbred lines for standardized areas and elution times

Inbred	Variance source	df	Peak percent area			Elution time		
			Mean square	Variance component	% of total variation	Mean square	Variance component	% of total variation
051	Peak	19	88.38	27.01	91.48	422,165.9	132,782.8	100
	Source/Peak	44	2.52	2.52	8.52	12.8	12.8	< 1
207	Peak	21	49.33	15.23	96.72	451,428.3	140,807.1	100
	Source/Peak	49	0.52	0.52	3.28	14.7	14.7	< 1
G35	Peak	20	19.91	7.63	95.47	334,544.3	130,617.9	100
	Source/Peak	33	0.36	0.36	4.53	17.3	17.3	< 1
G39	Peak	21	120.64	28.08	96.23	693,107.3	162,800.5	100
	Source/Peak	72	1.10	1.10	3.77	9.3	9.3	< 1
G50	Peak	26	35.19	15.99	90.49	340,251.4	162,341.9	100
	Source/Peak	30	1.68	1.68	9.51	12.9	12.9	< 1

Table 4. Analysis of variance and variance components over 5 inbreds for standarized areas and elution times

Variance source	df	Peak percent area			Elution time		
		Mean square	Variance component	% of total variation	Mean square	Variance component	% of total variation
Peak	26	92.77			1,800,803.8		
Inbred/Peak	85	49.30	16.16	93.0	22,529.9	7,568.7	99.8
Source/Peak/Inbred	228	1.21	1.22	7.0	12.8	12.8	0.2

from replicate extracts of two sources each of inbreds O51 and G39. In all cases, replicate extracts gave identical profiles. Comparisons of chromatograms from dissimilar sources of inbred O51 likewise revealed no differences. However, contrasts were apparent between chromatograms derived from two different sources of inbred G39 (Fig. 2). A minor peak eluting at approximately 20 min for inbred G39, source 5 (G39-5), was revealed as a major peak in sample G39-3 and additional minor peaks eluting at approximately 26 and 27 min were revealed in extracts from sample G39-3 alone.

Table 3 presents the results of analysis of variance among all sources for each of the five inbred lines listed in Table 1. Representative chromatograms of each source for inbreds 207 and G50 are displayed in Figs. 3 and 4, respectively. The percent variation in peak areas attributable to source effects (combined source effects and experimental error) ranged from 3.28% (inbred O51) to 9.51% (inbred G50). Percentage variation for elution times attributable to source effects was consistently < 1.0% (Table 4). Over all inbreds, the mean contribution of source to percent area and elution time variation, was 7 and 0.2%, respectively.

Representative chromatograms from four sources of inbreds 207 and G50 (Figs. 3 and 4) portray the consistency of zein profiles for seed produced across different locations and years. Contrasts were apparent only for minor peaks; those contributing 2.0% or less to the normalized chromatographic profile. For inbred G50, additional minor peaks eluting at approximately 6, 8, and 19 min were resolved in sample G50-1 and sample G50-4 revealed no peaks eluting after 25 min. Occasional variation in the presence or absence of peaks contributing > 2% of the profile was due to the incomplete resolution of peak shoulders and not to the absence of proteins (for example, a peak eluting at approximately 23 min in samples G50-3 and G50-4 compared to a shoulder for G50-1 and G50-2).

Discussion

It is well documented (Higgins 1984) that environmental effects can markedly affect, not only the quantitative, but also the qualitative constitution of proteins that are formed in the seed. Prolamin and protein profiles, as revealed by electrophoresis, have been shown to exhibit little or no environmental effects (Marchylo and LaBerge 1980; Lookhart and Finney 1984; Sarkar and Bose 1984). However, RP-HPLC separates proteins according to hydrophobicity and not, as with electrophoresis, according to net electrical charge or molecular size. Therefore, conclusions with regard to en-

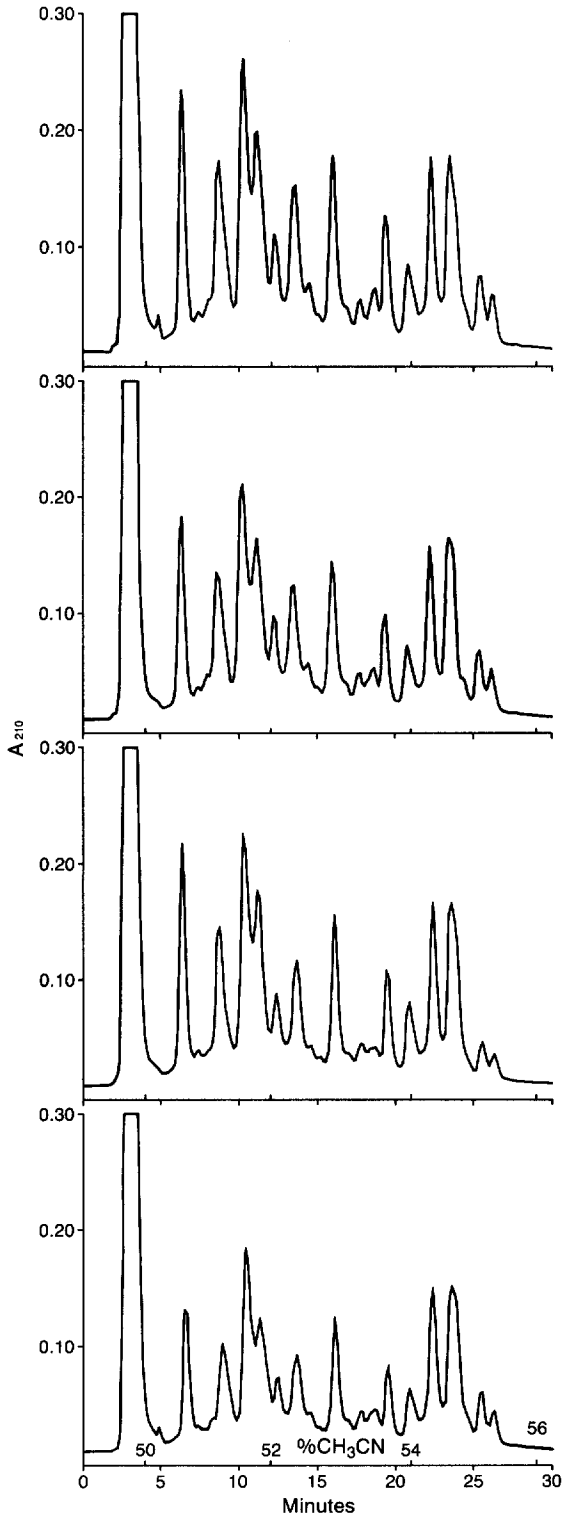

Fig. 3. Chromatograms of different sources of inbred 207. Sources 207-1 through 207-4 are arranged from bottom to top of figure

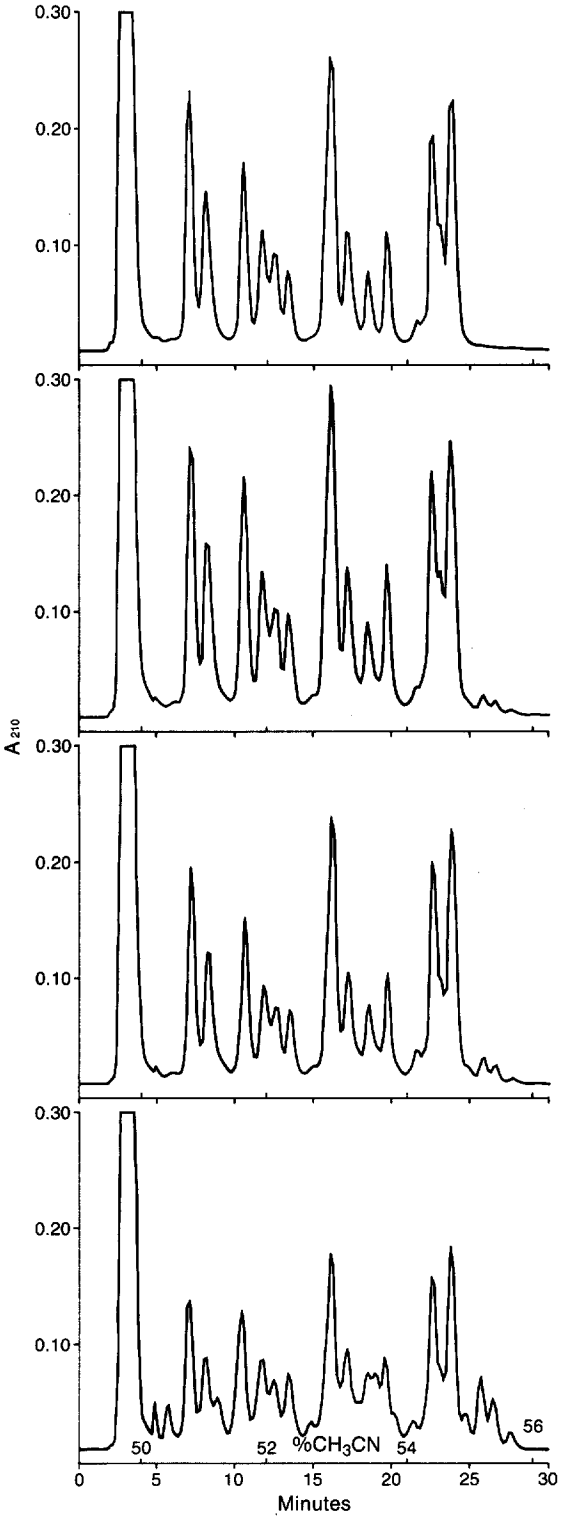

Fig. 4. Chromatograms of different sources of inbred G50. Sources G50-1 through G50-4 are arranged from bottom to top of figure

vironmental interactions that are based upon electrophoretic data could be inappropriate with respect to RP-HPLC analyses. Furthermore, the high resolution, both quantitative and qualitative, that is afforded by RP-HPLC demands a more thorough analysis of the stability of chromatographic profiles with regard to genotype.

Results from chromatographic separations of replicate extracts revealed that the variance components due to extraction and experimental error were relatively minor and thus the major contribution to variance between sources was due to source. Data from replicate extracts showed that differences between sources were real. However, data from all sources showed that the source contribution was relatively small. Although environmental effects upon seed of a given genotype could have a minor affect on peak area, the effects with regard to presence or absence of peaks were limited to peaks contributing 2% or less to the normalized profile. In some instances, contrasts were apparent in the quality of resolution of peak shoulders. However, observation of the chromatograms showed this to be the result of a relatively low sampling rate by the data recorder (1 signal/s) rather than the lack of any proteins. Quality of resolution should be enhanced by an increase in signal flow rate from the spectrophotometer to the data recorder to 3 or 5 signals/s.

These data show that although environmental effects upon zein chromatographic profiles cannot be ignored, they are relatively insignificant. These conclusions are based only on the inbred lines studied herein, but these lines do represent a broad array of U.S. maize germplasm. RP-HPLC of prolamin proteins can provide highly distinctive profiles of maize inbred lines that closely reflect genotype. Both the qualitative and quantitative aspects of these profiles could be used to establish line identity and to investigate germplasm relationships. RP-HPLC could also be used to provide additional insights into the genetic regulation and control of prolamin genes.

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References

Bietz JA (1983) Separation of cereal proteins by reversed-phase high-performance liquid chromatography. J Chromatogr 155:219–238

Bietz JA, Burnouf T, Cobb LA, Wall JS (1984) Wheat cultivar identification and genetic analysis by reversed-phase high-performance liquid chromatography. Cereal Chem 61:129–135

Bietz JA, Burnouf T, Cobb LA, Wall JS (1984b) Gliadin analysis by reversed-phase high-performance liquid chromatography; optimization of extraction conditions. Cereal Chem 61:124–129

Burnouf T, Bietz JA (1984) Reversed-phase high-performance liquid chromatography of durum wheat gliadins: relationships to durum wheat quality. J Cereal Sci 2:3–14

Burnouf T, Bietz JA, Cobb LA, Wall JS (1983) Chromatographie liquide haute performance des gliadines de variétés de Blé tendre (*Triticum aestivum* L.) cultivées en France et possibilités d'identification variétale. C R Acad Sci 297:377–382

Higgins TJV (1984) Synthesis and regulation of major proteins in seeds. Annu Rev Plant Physiol 35:191–221

Landry J, Paulis JW, Fey DA (1983) Relationship between alcohol-soluble proteins extracted from maize endosperm by different methods. J Agric Food Chem 31:1317–1322

Lookhart GL, Finney KF (1984) Polyacrylamide gel electrophoresis of wheat gliadins: the effect of environment and germination. Cereal Chem 61:496–499

Marchylo BA, Kruger JE (1984) Identification of Canadian barley cultivars by reversed-phase high-performance liquid chromatography. Cereal Chem 61:295–301

Marchylo BA, LaBerge DE (1980) Barley cultivar identification by electrophoretic analysis of hordein proteins. 1. Extraction and separation of hordein proteins and environmental effects on the hordein electrophoregram. Can J Plant Sci 60:1343–1350

Sarkar R, Bose S (1984) Electrophoretic characterization of rice varieties using single seed (salt soluble) proteins. Theor Appl Genet 68:415–419

Soave C, Solamini F (1983) Genetic organization and regulation of maize storage proteins. In: Daussant J, Mossé J, Vaughan J (eds) Seed proteins. Academic Press, London New York, pp 205–218

Wall JS, Fey DA, Paulis JW (1984) Improved two-dimensional electrophoretic separation of zein proteins: application to study of zein inheritance in corn genotypes. Cereal Chem 61:141–146

Wilson CM (1984) Isoelectric focusing of zein in agarose. Cereal Chem 61:198–200